Clin. exp. Immunol. (1985) **62,** 738–745.

Measurement of anti-cardiolipin antibodies by an enzyme-linked immunosorbent assay (ELISA): standardization and quantitation of results

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(Accepted for publication 26 June 1985)

SUMMARY

We describe the development of a simple and highly sensitive double antibody sandwich enzyme-linked immunosorbent assay (ELISA) for measuring IgG and IgM anti-cardiolipin antibodies (ACA).

Microtitre plates were coated with cardiolipin at a concentration of 45 μg/ml by evaporation under nitrogen. Non-specific binding of diluted sera was eliminated by blocking of plates with 10% fetal calf serum in phosphate buffered saline (PBS/FCS) for 2 h. Then sera (100 μl) at a dilution of 1:100 were incubated in the wells for 1 h. Affinity purified goat anti-human IgG or IgM (100 μl) at a concentration of 1 μg/ml was subsequently added and allowed to incubate for 1 h; detection of ACA was achieved using an alkaline phosphatase conjugated rabbit anti-goat IgG reagent by reading the colorimetric yield at 405 nm after incubation with substrate. Reference serum pools were established to study reproducibility of the assay throughout its sensitivity range, and Standard curves were established. The quantitative normal range was 0–9·0 Anti-cardiolipin ELISA Units (AEU) for IgG and 0–8·0 (AEU) for IgM–ACA. A strong correlation was found between the ELISA and radioimmunoassay methods for measuring ACA of both IgG and IgM classes.

Results from 65 patients with systemic lupus erythematosus (SLE) and 45 patients with seropositive rheumatoid arthritis are also reported. The advantages of the ELISA method for quantitative determination of ACA levels, should make it a useful and reliable method for clinical and experimental monitoring of patients with SLE and associated autoimmune disorders.

Keywords anticardiolipin ELISA standardization antiphospholipid antibodies

INTRODUCTION

Of the large numbers of autoantibodies described in patients with systemic lupus erythematosus (SLE) and associated autoimmune disorders, anti-phospholipid antibodies have received scant attention until comparatively recently.

With the development of sensitive methods for the estimation of various anti-phospholipid antibodies (amongst which anticardiolipin antibodies are the most frequently detected) (Harris *et al.*, 1983; Koike *et al.*, 1984; Norberg *et al.*, 1984; Colaco & Male 1985; Harris *et al.*, 1985a), the

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clinical associations of these antibodies with thromboembolic disease (Harris *et al.*, 1983) thrombocytopenia. (Harris *et al.*, 1985b) and autoimmune thrombocytopenic purpura (Harris *et al.*, 1985c) have become apparent.

Methods for measurement of these antibodies in serum have generally concentrated to date on arbitrary estimations of anti-phospholipid antibody levels, without emphasis on validation and standardization of the assays used. As enzyme-linked immunosorbent or ELISA assays (Engvall & Perlman, 1971) are now increasingly being applied to the detection of antibodies in a variety of biological substances, and their potential as reliable sensitive reproducible and rapid assays is well established, this technique has been applied recently to the measurement of anti-phospholipid antibodies (Koike *et al.*, 1984), although only IgG class ACA were sought and the assay was a single antibody method.

We report the development of a sensitive double antibody ELISA assay for the detection of both IgG and IgM classes of anti-cardiolipin antibodies. We have tried to establish appropriate values for normal and abnormal levels and we have established units for future reporting of anti-cardiolipin antibody levels, which we hope will prove useful in routine screening for the presence of, and in the serial monitoring of these antibodies in the sera of patients. These units will also permit comparison of results from different centres and may assist in the standardization of ELISA ACA assays.

MATERIALS AND METHODS

Materials were obtained as follows: Titertek rigid polystyrene plates (350 μl capacity flat bottom wells) which were used as the solid phase (Flow Laboratories, Irvine, Scotland). Cardiolipin in ethanol, rabbit anti-goat IgG (whole molecule) alkaline phosphatase conjugate, and p-nitrophenyl phosphate—Sigma substrate 104, (Sigma Ltd, Poole, UK). Affinity purified goat anti-human IgG and IgM from (Tago Inc., Burnigame, California, USA). Analar grade diethanolamine, magnesium chloride hexahydrate, sodium hydroxide, and sodium azide (BDH, UK), phosphate buffered saline tablets (PBS) (Oxoid Ltd, Basingstoke, UK).

Controls and patients. Control sera were obtained from 135 healthy subjects, (86 females and 49 males).

Sera were also obtained from 65 patients with diagnosed SLE, from 45 patients with sero-positive rheumatoid arthritis, one ANA positive patient with Felty's syndrome, and one patient with mixed essential type II cryoglobulinaemia.

Development of ELISA protocol. The final ELISA conditions described later on in this study were decided after several experiments to determine (a) the optimum antigen concentration for coating the plates and (b) the optimum, first antibody and antibody conjugate concentrations. Variation of assay temperature, and differing incubation times for each step were also studied. The optimum conditions required for a 1 day assay consisted of: (a) blocking of the plates with 10% fetal calf serum in PBS (PBS/FCS) for 2 h at room temperature, (b) incubation with patient serum for one hour at room temperature, (c) incubation with enzyme conjugate for 1 h at room temperature and incubation with enzyme substrate for 1 h at 25°C in a constant temperature cabinet.

The blank value, estimated in triplicate on each plate was obtained by identical treatment of wells using 100 μl of PBS/FCS, in place of serum.

Positive and negative sera were measured in the assay to obtain dilution curves for both IgG and IgM classes of anticardiolipin antibodies. From this a 1/100 serum dilution was chosen as the routinely used dilution in the assay.

Final ELISA Protocol. Cardiolipin (30 μl) at a concentration of 45 μg/ml in ethanol was added to titertek polystyrene plates and coated onto the plate surface by evaporation under nitrogen. Plates were then blocked to prevent non-specific binding of immunoglobulins by the addition of 110 μl of PBS/FCS for 2 h at room temperature. They were then washed four times with 120 μl PBS, soaking for 2 min between each wash. Serum or standard (100 μl) diluted 1:100 in PBS/FCS (in triplicate) was added to test wells and 100 μl of PBS/FCS was added to the blank control wells. The plates were incubated for 1 h at room temperature and washed four times with PBS as previously described.

First antibody (100 μl) goat antihuman IgG or IgM diluted 1:4000 in PBS/FCS was added to each well and the plates were again incubated for 1 h at room temperature and then washed four times as before. The alkaline phosphatase-conjugated second antibody (rabbit anti-goat IgG) was diluted 1:1000 in PBS/FCS and 100 μl aliquots were added to each well and incubated for 1 h at room temperature, after which time the plates were again washed four times with 120 μl/well PBS; p-nitrophenyl phosphate (1 mg/ml) was prepared immediately before use in diethanolamine buffer (pH 9·8) and 100 μl aliquots were added to each well and the plates were incubated in the dark at 25°C for 1 h. The reaction was stopped by the addition of 3M NaOH (50 μl) to all wells and the optical absorbance of each well was read at 405 nm with a Titertek Multiscan (Flow Laboratories, Irvine, UK). A synopsis of the ELISA procedure used in this study, is shown in Fig. 1 in the form of a flow chart.

Characterization of the method. Antibody response curves were prepared by dilution of positive samples in a negative sample (Fig. 2). Samples were diluted with normal serum at doubling dilutions from 1:1 to 1:256 and were then tested in the assay at 1:100 dilution with PBS/FCS. These serial dilutions of positive sera in a negative serum were performed to provide sequentially defined antibody activity, at both high and low IgG and IgM ACA levels.

Determination of specificity of antibody binding. To prove that the antigenic determinant recognized in this test was cardiolipin, the following experiment was conducted.

Aliquots of highly positive samples for IgG and IgM anti-cardiolipin antibody were incubated

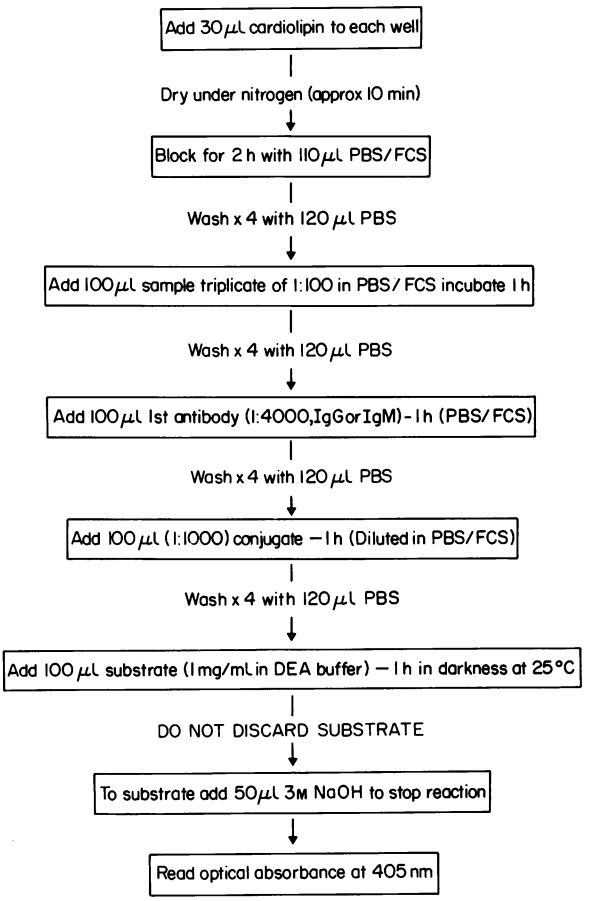

Fig. 1. Flow chart showing the different stages in the anti-cardiolipin ELISA assay. All incubations at room temperature unless stated otherwise.

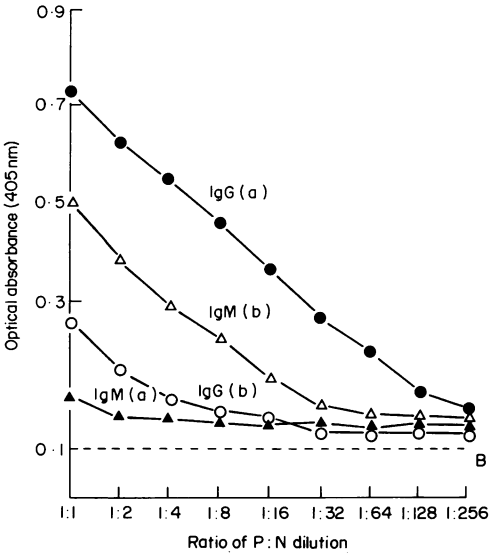

Fig. 2. Antibody response curves for IgG and IgM anti-cardiolipin levels (expressed as optical absorbance at 405 nm) obtained by dilution of highly positive samples (P), in a negative sample (N) and assayed at 1:100 dilution in PBS/FCS in the ELISA assay. The absorbance for the diluent blank value (– – –) is also shown. (●,▲) patient 1; (○,△) patient 2; (●,○) IgG; (▲,△) IgM.

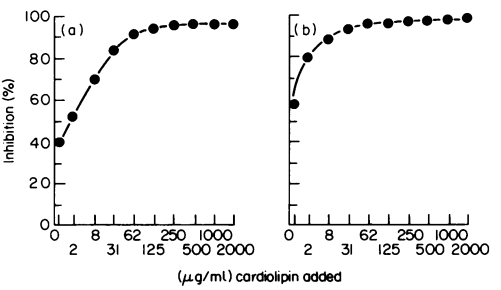

Fig. 3. Inhibition of IgG(a) and IgM(b) anti-cardiolipin antibody levels by incubation with pure cardiolipin at the concentrations indicated. The presence of ACA was then assessed according to the method described in materials and methods.

with pure cardiolipin at concentrations ranging from 0·5 to 2,000 μg/ml. Antibodies to cardiolipin of either class were determined in the ELISA assay, where an almost total inhibition of antibody, binding was demonstrated, at concentrations above 31 μg of added cardiolipin in both the IgG and IgM assays (Fig. 3). Inhibition results were expressed as percentage inhibition of cardiolipin binding activity and calculated as shown below with prior blank subtraction:

$$\text{Inhibition (\%)} = \frac{\text{OA of uninhibited sample} - \text{OA of inhibited sample}}{\text{OA of uninhibited sample}} \times 100$$

These results are consistent with the detection of IgG and IgM antibodies with specificity for cardiolipin.

Anticardiolipin radioimmunoassay. Anticardiolipin antibodies for IgG and IgM were determined by RIA (Harris *et al.*, 1983).

Latex agglutination. The test was carried out with the Ortho RA Test Kit (Ortho Diagnostics, Beerse, Belgium). Titres of $< 1:40$ were considered to be negative.

DNA-binding assay. DNA-binding was determined by the Farr-technique (Pincus *et al.*, 1969), where values for DNA-binding $< 30\%$ were considered to be negative.

Statistical analysis. Correlation coefficients and unpaired *t*-tests were used when appropriate.

Reproducibility. To determine the reproducibility of the ELISA assay, throughout its sensitivity range, four serum pools (I–IV) were created, with anti-cardiolipin antibody levels which covered the entire range of the assay. Results of anti-cardiolipin levels for these pools were similar by both the ELISA and RIA assays determined in our and other laboratories.

Each pool was included in every assay and on every plate in triplicate over a period of 6 months. Results were expressed as a Binding Index (BI) calculated from optical absorbance (OA) values at 405 nm as follows:

$$\text{BI} = \frac{\text{OA (Test sample)} - \text{OA (Blank)}}{\text{OA (Reference normal pool I)} - \text{OA (Blank)}}$$

The reference normal pool (Pool I) is composed of pooled ACA negative sera from 135 healthy individuals. This method of expressing results compared to a normal reference pool, compensates for day to day variations in the assay. These depend on the exact timing of the various incubation steps, fluctuations in room-temperature and reagent dilution etc.

RESULTS

Intra-assay coefficients of variation were obtained by testing the same pooled serum samples on the same plate twelve times were found to be 4·4% (Pool II), 4·8% (Pool III) and 5·7% (Pool IV), for IgG and 11·9% (Pool II), 12·9% (Pool III) and 11·1% (Pool IV) for IgM anticardiolipin antibodies.

Inter-assay coefficients of variation expressed in BI were calculated from 24 different assays during a period of 4 months. These were 9·3% (Pool II), 10·4% (Pool III) and 8·9% (Pool IV) for IgG and 15·4% (Pool II), 10·9% (Pool III) and 13% (Pool IV) for IgM anticardiolipin antibodies respectively.

Normal Controls

Sera from 86 normal females and 49 normal males were tested for both classes of ACA. The results are summarized below and mean values are given for both the OA value and BI for purposes of comparison.

A positive value is defined as an OA or BI > 5 standard deviations above the mean normal control value of the 135 normal subjects. Thus for the normal subjects studied, the mean value for BI was $1{\cdot}042 + 0{\cdot}406$ s.d. (mean value for O.A. $= 0{\cdot}050 + 0{\cdot}022$ s.d.), for IgG and for IgM BI was $1{\cdot}026 \pm 0{\cdot}578$ s.d. (mean value for O.A. $= 0{\cdot}0450 + 0{\cdot}025$ s.d.). A positive value for IgG was a BI $> 3{\cdot}072$ (O.A. $> 0{\cdot}159$); for IgM a positive value was a BI $> 3{\cdot}916$ (O.A. $> 0{\cdot}168$).

Comparison of ELISA with RIA

Sera from 48 unselected patients attending the Rheumatology Out-Patients Clinic were assayed for anticardiolipin antibodies by the ELISA method and by the RIA method of (E. N. Harris *et al.*, 1983). Results for both the IgG and IgM ACA classes as obtained by the two methods are shown in Fig. 4. Results are shown as cpm for the RIA and as O.A. at 405 nm for the ELISA. Strong positive correlations between the two methods were shown for both the IgG ($r = 0{\cdot}832$) and IgM ($r = 0{\cdot}856$) classes of antibody.

Patients

To assess the possibility of cross-reactivity between anti-dsDNA antibodies and anticardiolipin antibodies 65 SLE patients with DNA-binding values ranging from 0–98% (Farr-assay) were checked in the ACA ELISA assay for the presence of both classes of ACA antibodies. Of the 65 patients 19 (29·2%) were positive for IgG and 18 (27·7%) for IgM ACA. No correlation was seen between levels of ACA and DNA.

In a further study designed to assess cross-reactivity between ACA and rheumatoid factor

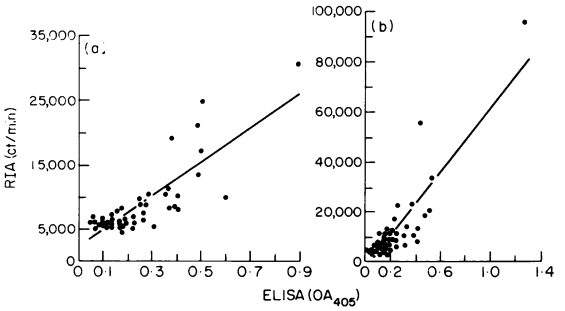

Fig. 4. Comparison of ELISA and RIA for IgG(a) and IgM(b) anticardiolipin antibodies. Sera from unselected patients were assayed for the presence of ACA by ELISA and RIA. All results from the ELISA assay are presented as optical absorbance (OA) and for RIA as counts per minute (ct/min). Strong correlations were found for both IgG ($r=0{\cdot}832$) and IgM ($r=0{\cdot}856$) ACA.

(Latex agglutination titres), sera from 47 patients, of whom 45 had rheumatoid arthritis, one patient had Felty's syndrome with a positive ANA and normal DNA binding, and one patient with type II mixed essential cryoglobulinaemia (latex titre 1:20,000) were studied. No correlation was found between rheumatoid factor titre and ACA antibodies of either immunoglobulin class. Out of the 47 patients one was positive for IgG-ACA and a different patient was positive for IgM-ACA.

Quantitation of ACA ELISA assay

After the establishment of the ELISA system used in the present study, a need for quantification of ACA levels and for standardization of ACA levels between different centres emerged. From ELISA results which represented the entire spectrum of normal and pathological anti-cardiolipin levels, 55 sera for IgG-ACA and 48 sera for IgM-ACA were selected for further study. Each serum was titrated until its BI was normal (i.e. $<$mean$+5$ s.d.). Titre end-points were then replotted as a function of BI readings (obtained at the serum working dilution (1:100)) at the positive/negative cut-off point, to produce standard curves for both classes of antibody. (Malvano *et al.*, 1982).

Units designated as 'anti-cardiolipin ELISA units' (AEU) were derived by converting the logarithmic scale of reciprocal of dilution to AEU. The resultant normal range was 0–9 AEU for IgG and 0–8·0 AEU for IgM anti-cardiolipin antibodies.

Subsequently pooled samples at seven different points on each curve covering the entire range of ACA levels were chosen to create two separate standard curves one for IgG and one for IgM ACA standards. These pools were aliquoted in 10 μl amounts into sets of seven standards in tubes stored at $-70°$C and they are used routinely at a (1:100) dilution in each assay. Thus for each assay

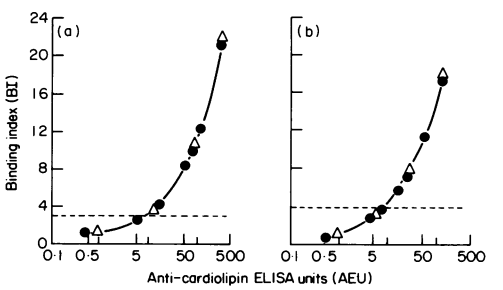

Fig. 5. Standard curves for IgG(a) and IgM(b) anticardiolipin antibody levels, relating B.I. values (ordinate), to Anticardiolipin ELISA Units (abscissa). Results shown are means of 10 standard curves (●) as obtained on 10 successive occasions. Results for reference pools (I–IV) are also shown (△). (– – –) indicates the upper limit of normal (5 × s.d.).

following the creation of the pooled standards, one set of standards for each class of antibody was thawed out, diluted with PBS/FCS and assayed in triplicate on each plate in addition to the established reference pools. (I–IV).

Results of means for a series of standard curves and reference pools (I–IV) run in 10 consecutive assays over a period of 2 months are shown in Fig. 5.

Expressed in this manner the highest level of serum ACA found was 325 AEU for IgG and 175 AEU for IgM anti-cardiolipin antibodies.

DISCUSSION

Considerable interest and research developments in ELISA assays has recently led to their transition from research to the clinical laboratories, for routine diagnostic investigations. ELISA assays have the advantages of providing measurements which are as sensitive as radioimmunoassay without the hazards of radioactive materials. Since ELISA assays are specific for immunoglobulin binding, they directly measure the quantity of antibody capable of reacting with a specific antigen without the possibility of interference by non-immunoglobulin binding material. These assays are quicker and cheaper than radioimmunoassays and enable large numbers of samples to be tested in one batch and in one day, while using very small quantities of serum.

A number of methods have been published for the measurement of anti-cardiolipin antibodies, including radioimmunoassays (Harris *et al.*, 1983; Harris *et al.*, 1985, a; Colaco & Male, 1985) and recently ELISA assays (Koike *et al.*, 1984; Norberg *et al.*, 1984).

None of the assays on anti-cardiolipin antibody levels published so far has studied a sufficiently large group of normals. Their normal limits therefore are arbitrary and are not statistically satisfactory. We have established normal limits, by studying 135 normal subjects, and have chosen an upper limit of normal, which is sufficiently high to exclude over 99·9% of normals. Using the BI method of expressing results, the distribution of normal controls in the present study was found to be normal, reinforcing further the validity of our cut-off point (Reed, Henry & Mason, 1971).

In the experiment designed to investigate the specificity of antibody binding, the results are consistent with the detection of IgG and IgM antibodies with specificity for cardiolipin. If the antigen recognized by the antibodies detected in our assay was not cardiolipin, inhibition by pure cardiolipin would not have been seen in the quantitative fashion observed.

In the two groups of patients studied (SLE & RA) no cross-reactivity between anti-dsDNA antibodies or latex rheumatoid factor titres was seen with either class of anti-cardiolipin antibodies. The incidence of positive patients in SLE (29·2% for IgG, 27·7% IgM), as well as for rheumatoid arthritis (2·2% for IgG or IgM) was slightly lower than those reported previously (Harris *et al.*, 1983; Koike *et al.*, 1984; Colaco & Male, 1985). This discrepancy is mainly due to the difficulty in defining a positive level, as in previous studies positive results were based on different methods. Harris *et al.* (1983) chose 2·76 standard deviations above the mean normal level as the upper limit of normal and included 30 control subjects, Koike *et al.* (1984) expressed their results in terms of a percentage above the level of a standard serum and only studied 24 SLE sera and Colaco & Male (1985), who obtained the greatest positive percentage rate (73/87 for IgM = 84%, and 71/87 for IgG = 82% in SLE patients) set their upper limit of normal as only two standard deviations above that of the mean of 23 normal controls.

In an effort to standardize our ELISA assay we have created standards and units for the quantitative reporting of both IgG and IgM anti-cardiolipin antibody levels, in the way suggested by de Savigney (1980), as the best method of expressing ELISA results. The creation of a large number of standards eliminates any errors likely to arise from inaccuracies in aliquoting and repeated thawing, and enables very accurate measurement of serial levels of ACA in patient samples.

We wish to thank Dr A. E. Gharavi for his invaluable help with the radioimmunoassay, and Miss Selina Lydon for her expert secretarial assistance.

REFERENCES

COLACO, C.B. & MALE, D.K. (1985) Antiphospholipid antibodies in syphilis and a thrombotic subset of SLE: distinct profiles of epitope specificity. *Clin. exp. Immunol.* **59,** 449.

DE SAVIGNY, D. (1980) The communication of ELISA data from laboratory to clinician. *J. Immunoassay.* **1(i),** 105.

ENGVALL, E. & PERLMANN, P. (1971) Enzyme linked immunosorbent assay (ELISA): Quantitative assay of IgG. *Immunochem.* **8,** 871.

HARRIS, E.N., GHARAVI, A.E., BOEY, M.L., PATEL, B.M., MACKWORTH-YOUNG, C.G., LOIZOU, S. & HUGHES G.R.V. (1983) Anticardiolipin antibodies: detection by radioimmunoassay and association with thrombosis in systemic lupus erythematosus. *Lancet*, **ii,** 1088.

HARRIS, E.N., GHARAVI, A.E., LOIZOU, S., DERUE, G., CHAN, J.K., PATEL, B.M., MACKWORTH-YOUNG, C.G., BUNN, C.C. & HUGHES, G.R.V. (1985a) Crossreactivity of antiphospholipid antibodies. *J. clin. Lab. Immunol.* **16,** 1.

HARRIS, E.N., ASHERSON, R.A., GHARAVI, A.E., MORGAN, S.H., DERUE, G. & HUGHES, G.R.V. (1985b) Thrombocytopenia in SLE and related autoimmune disorders: association with anticardiolipin antibodies. *Br. J. Haematol.* **59,** 227.

HARRIS, E.N., GHARAVI, A.E., HEDGE, U., DERUE, G., MORGAN, S.H., ENGLERT, H., CHAN, J.K., ASHERSON, R.A. & HUGHES, G.R.V. (1985c) Anticardiolipin antibodies in autoimmune thrombocytopenic purpura. *Br. J. Haematol.* **59,** 231.

KOIKE, T., SUEISHI, M., FUNAKI, H., TOMIOKA, H. & YOSHIDA, S. (1984) Antiphospholipid antibodies and biological false positive serological test for syphilis in patients with systemic lupus erythematosus. *Clin. exp. Immunol.* **56,** 193.

MALVANO, R., BONIOLO, A., DOVIS, M. & ZANNINO, M. (1982) ELISA for antibody measurement: aspects related to data expression. *J. Immunol. Meth.* **48,** 51.

NORBERG, R., GARDLUND, B., THORSTENSSON, R. & LIDMAN, K. (1984) Further immunological studies of sera containing antimitochondrial antibodies, type M5. *Clin. exp. Immunol.* **58,** 639.

PINCUS, T., SCHUR, R.H., ROSE, J.A., DECKER, J.L. & N TALAL, (1969) Measurement of serum DNA-binding activity in systemic lupus erythematosus. *N. Engl. J. Med.* **281,** 701.

REED, A.H., HENRY, R.J. & MASON, W.B. (1971) Influence of statistical method used on the resulting estimate of normal range. *Clin. Chem.* **17,** 275.